

COMBINATIONS AND METHODS FOR PROMOTING *IN VIVO* LIVER CELL PROLIFERATION
AND ENHANCING *IN VIVO* LIVER-DIRECTED GENE TRANSDUCTION

FIELD OF THE INVENTION

5 This invention relates to combinations and methods for inducing a semi-synchronous wave of liver cell proliferation *in vivo* and for combinations and methods for inducing a semi-synchronous wave of liver cell proliferation and transduction of proliferating liver cells *in vivo*.

BACKGROUND

10 Several characteristics make the liver an attractive target organ for the application of gene therapy. First, the liver is the situs of essential metabolic pathways, and consequently it is often the organ involved in genetic metabolic diseases. Data from model animals suggested that expression of a functional version of a gene in the liver of the affected animal can substantially restore metabolic function. Recent results obtained in human clinical trials have supported this view. The liver is also intimately associated with the circulatory system, and can serve as a
15 secretory organ for the systemic delivery of therapeutic proteins or polypeptides. Another advantage the liver presents as an organ for gene therapy is the particular structure of its endothelium, which permits access of large molecules to the liver parenchyma. Vectors commonly used in gene therapy therefore can penetrate the organ and reach the liver cells.

20 Liver gene therapy has broad applicability, and can be used in the treatment and prevention of conditions by expression of RNAs, polypeptides or proteins, or combinations thereof, in the liver for action in the liver or secretion of polypeptides or proteins into the bloodstream or the gastrointestinal tract. Conditions that may be treated or prevented by liver gene therapy include, for example, inborn errors of metabolism, lymph and blood protein and

polypeptide deficiencies, including for example, clotting abnormalities, obesity, hypercholesterolemia, cystic fibrosis, alpha 1-antitrypsin deficiency, phenylketonuria, diabetes, Wilson disease, ornithine transcarbamylase deficiency, among others, cancer, several forms of hepatitis and other infectious diseases affecting liver, hypertension, cirrhosis of the liver, among
5 others.

Preventive and therapeutic applications of gene transfer to the liver have been recently reviewed in the literature. See, e.g., Davern, et al., Dig. Dis. 16:23-37 (1998); Forbes and Hodgson, Aliment. Pharmacol. Ther. 11:823-836 (1997); Chang and Wu, Gastroenterology 106:1076-1084 (1994); and Horwich, Curr. Top. Microbiol. Immunol. 168:185-200 (1991).

10 Some liver-directed gene therapy applications require long-term moderate to high level expression of a product encoded by a gene construct. To that end, it has been found desirable that the vector transduce a large proportion of target cells, and that the gene construct stably integrate into the genome of the transduced target cells. Transduction of gene constructs into the genome of liver cells has been accomplished by several means, including the use of retroviral
15 vectors, adeno-associated virus vectors, lipofectant agents, receptor-based transfer, and combinations thereof. See, e.g., Sandig and Strauss, J. Mol. Med. 74:205-212 (1996). Cell division, in particular passage of a cell through the S phase of the cell cycle, has been found to greatly increase, and in some cases has been found indispensable, for integration of a transduced gene construct into a cell's genome or for its adequate expression. See, e.g., Roe, et al., EMBO J.
20 12:2099-2108 (1993)(retroviruses) and Fisher, et al., Nat. Med. 3:306-312 (1997)(adeno-associated virus). The induction of liver cells to proliferate has also been found to present other advantages for liver-directed gene transfer, including the up-regulation of viral receptor

expression by dividing liver cells, which has a positive effect on the efficiency of retroviral vector gene transfer into liver cells. Ott, et al., J. Biol. Chem. 273(19):11954-61 (1998).

Retroviral vectors provide several advantages for *in vivo* gene transfer, including their ability to carry relatively large amounts of genetic material (8 kilobases (kb)) and their reliable, efficient and consistent integration into a target cell's genome. The use of recombinant retroviruses as gene transfer vectors into liver cells has nevertheless been limited due to methodological difficulties. The well documented quiescent state of liver cells under normal conditions has prevented efficient *in vivo* liver cell transduction by retroviral vectors because retroviral vectors require division of target cells within a few hours of vector entry for stable integration of the gene construct into the genome of the target cell.

While techniques have been described for inducing the proliferation and transduction of liver cells *in vivo* in model animals, these techniques have been found inadequate for general use. One presently used strategy, for example, depends on liver regeneration induced by removal of a large portion of the liver (Ferry, et al., Proc. Nat'l Acad. Sci. USA 88:8377-8381 (1991)), by causing apoptosis (cell death) of a large portion of the liver by portal branch occlusion (Bowling, et al., Human Gene Therapy 7:2113-2121 (1996)), or by producing severe liver damage by expression of toxic genes in liver cells (Lieber, et al., Human Gene Therapy 6:1029-37 (1995)). These techniques are laborious, intrusive and may result in complications due to the rapid elimination of a large portion of the liver of an individual. The need for major surgery before integrative transfer is clinically undesirable. Moreover, these techniques are particularly unsuitable for individuals in need of gene therapy due to an already present liver insufficiency.

Less invasive procedures for inducing replication of liver cells have been described, but these procedures have failed to solve the existing problem of low transduction, as they have been

found to induce a low number of liver cells to proliferate, and generate an inadequately low proportion of transduced cells. For example, Bosch, et al., (J. Clin. Invest. 98:2683-2687 (1996)) reported that administration of keratinocyte growth factor will induce the proliferation of liver cells. However, only 2% of the hepatocytes in the liver were transduced when a retroviral vector was administered 24 hours after KGF administration.

Kosai, et al., (Human Gene Therapy 9:1293-1301 (1998)) reported that hepatocyte growth factor administration followed by retroviral vector administration will increase transduction of liver cells *in vivo*. However, low transduction values of 1.3% were achieved. Similarly, Forbes, et al., (Gene Therapy 5:552-555 (1998)) reported that tri-iodothyronine administration induced proliferation of liver cells *in vivo*, and when followed by retroviral administration increased transduction of liver cells *in vivo*. Again, however, low transduction values of about 1.3% were achieved.

Therefore, it is desired to provide less invasive and more efficient, effective and convenient compositions and methods for inducing a semi-synchronous wave of liver cell proliferation and for the transduction of an adequate quantity of liver cells without the need for hepatectomy of a large portion of the liver, or otherwise eliminating a large number of liver cells by either expressing a toxic gene in the liver or by causing the apoptotic death of a large number of liver cells by partial portal occlusion.

SUMMARY OF THE INVENTION

In a first, independent aspect of the present invention, a combination for inducing a semi-synchronous wave of liver cell proliferation *in vivo* includes KGF and T3.

In a second, independent aspect of the present invention, concurrent administration of an effective amount of T3 and KGF will synergistically induce a semi-synchronous wave of liver

cell proliferation *in vivo*.

In a third, independent aspect of the present invention, *in vivo* induction of a semi-synchronous wave of liver cell proliferation using a combination including T3 and KGF prior to administration of a liver-directed gene transfer vector enhances transduction of liver cells *in vivo*.

5 In a fourth, independent aspect of the present invention, the concurrent administration of an effective amount of T3 and KGF, and the administration of a liver-directed gene transfer vector having an appropriate gene construct, is effective to express specific RNAs, proteins or polypeptides in the liver, or for the systemic delivery of proteins or polypeptides that may be accomplished from their production and secretion from the liver into the circulatory system or
10 the gastrointestinal tract.

In a fifth, independent aspect of the present invention, an organ including liver cells induced to proliferate by the concurrent administration of an effective amount of T3 and KGF and transduced by a liver-directed gene transfer vector is disclosed.

BRIEF DESCRIPTION OF THE DRAWINGS

15 Figure 1 is a bar graph representing the time course of hepatocyte proliferation *in vivo* following a single administration of KGF (1 mg/kg). Animals were sacrificed at 6 hour intervals, one hour prior to sacrifice all rats were injected with BrdU. Hepatic BrdU incorporation was detected in 4 μ m sections by a monoclonal antibody, as described in detail in the examples *infra*. The proportion of BrdU labeled nuclei was quantified from 2000 hepatocytes per section.

20 Results represent mean \pm S.D. "***" indicate a $p < 0.05$.

Figure 2 is a bar graph representing *in vivo* hepatocyte proliferation at 24 hours following a single and combined growth factor stimulation. Groups of four rats were administered either a single growth factor or a combination of two growth factors. A group of three rats received

injections of the growth factor diluents only. T3 (4 mg/kg) and KGF (1 mg/kg) were administered at 0 hours. All animals received BrdU at 23 hours and were killed at 24 hours. Hepatic BrdU incorporation was analyzed as described in detail in the examples *infra*. Hepatic BrdU incorporation was detected in 4 μ m sections by a monoclonal antibody. Results represent mean \pm S.D. "***" indicate a $p < 0.05$.

Figure 3 is a bar graph representing the cumulative proliferation measured following growth factor stimulation. T3 (4 mg/kg) together with KGF (1 mg/kg) were injected subcutaneously into a group of 3 rats. Two rats were injected with vehicle only. BrdU was injected at five hourly intervals between 12 and 32 hours in order to label all hepatocytes entering S-phase. Animals were killed at 33 hours and analysis of BrdU was made as described in detail in the examples *infra*. Result represent mean \pm S.D. "***" indicate a $p < 0.05$.

Figure 4 is a bar graph representing the proportion of hepatocytes expressing β -galactosidase at 9 days following intraportal injection of the TELCeB/AF-7 retrovirus. Groups of four rats received injections of either a single or a combination of growth factors. A group of three control rats received injections of the growth factor diluents only. At 24 hours all rats were injected intraportally with 1 ml of retrovirus during temporary hepatic artery and portal vein occlusion. All rats were sacrificed 9 days after vector administration. The proportion of hepatocytes expressing β -galactosidase was counted from 2000 hepatocytes in three different areas, as described in detail in the examples *infra*. Results represent mean \pm S.D. "***" indicate a $p < 0.05$.

Figure 5 is a bar graph representing the number of cells transduced following growth factor induced hyperplasia and peripheral venous administration of the retroviral vector. Three groups of 4 rats received an injection of either T3 or KGF, or both growth factors. Two rats were

injected with diluent only. All rats received 3 tail vein injections of 1 ml of the TELCeB/AF-7 vector at 18, 24 and 30 hours and were killed at day 10. All animals were sacrificed at day 10 and hepatic β -galactosidase expression was quantified as described in detail in the examples *infra*. Results represent mean \pm S.D. "***" indicate a $p < 0.05$.

5 DETAILED DESCRIPTION OF THE INVENTION

As used herein, "organ" means a discrete group of cells related by function, as may be used, for example, for transplantation or in a life support system, or the like.

As used herein, time is measured from the first administration of a growth factor to the individual, and is considered "hour 0" of "day 1."

10 As used herein, "semi-synchronous" means that although the cells that are induced to proliferate do not enter S-phase at exactly the same time, they do so relatively contemporaneously, such that the first induced cell divisions and the last induced cell divisions occur within a period of between about 0 hours and about 28 days, depending on the species, weight, and other variables affecting induced liver cell proliferation and the quantity and the
15 regimen of factor administration.

As used herein, "tri-iodothyronine" or "T3" refers to thyroid hormone or its analogs, and also includes compounds that bind and activate the thyroid hormone nuclear receptor.

The timing, dosage and mode of T3 administration should be determined by the prescribing physician or veterinarian, and may vary depending on mode of administration, the
20 species, age, and condition of the individual and in accordance with the needs of the individual and the time schedule of administration of the other factors. T3 may administered at any effective time before entrance of liver cells into S-phase is desired. T3 administration may also continue thereafter. Generally, however, T3 may be administered between about 0 and about 28

days before entrance of liver cells into S-phase is desired. Preferably T3 is administered between about 6 hours and about 14 days before entrance of liver cells into S-phase is desired. Most preferably, T3 is administered between about 24 hours and about 8 days before entrance of liver cells into S-phase is desired. More than one administration of T3 may be desirable in accordance with the needs of the individual as determined by the prescribing physician or veterinarian, and T3 may be administered any effective amount of times. Successive administrations generally can be performed at intervals ranging from about hourly to about weekly, but are preferably done at about daily intervals, or by continuous infusion. T3 administration may continue for any effective time after liver cells have begun to proliferate.

T3 may be administered in any effective daily dosage. In general, however, daily dosages will range from about 80 μg of T3 per kg of body weight per day ($\mu\text{g/kg}$) to about 80 mg/kg. Preferably, T3 is administered in daily dosages ranging from about 400 $\mu\text{g/kg}$ to about 40 mg/kg. Most preferred is the administration of daily dosages of about 4 mg/kg of T3. T3 may be administered by any effective route. It may be administered, for example, intravenously ("IV"), intramuscularly ("IM"), intraperitoneally, directly into the liver, subcutaneously, orally, inhaled, or by suppository. Subcutaneous administration is preferred. For subcutaneous administration, T3 may be dissolved in an appropriate volume of 0.01 M NaOH in 0.9 M NaCl, or any other effective carrier, and administered as a bolus. The formulation of T3 for administration is well known in the art.

As used herein, "keratinocyte growth factor" or "KGF" refers to the keratinocyte growth factor polypeptide or one of its analogs, or alternatively an active fragment of keratinocyte growth factor or one of its analogs, or a factor that binds and activates the keratinocyte growth factor receptor. Preferred is the recombinantly produced form of KGF. See, e.g., U.S. Patent

No. 5,731,170, and PCT Application No. WO 90/08771, published August 9, 1990 (directed to full length forms of KGF and variants); and PCT Application No. WO 96/11949, published April 25, 1996; PCT Application No. WO 96/11951, published April 25, 1996; and PCT Application No. WO 98/24813, published June 11, 1998 (directed to stable analogs of KGF) all of which are
5 incorporated herein by reference in their entirety, including figures.

The timing, dosage and mode of KGF administration should be determined by the prescribing physician or veterinarian, and may vary depending on the mode of administration, the species, age, and condition of the individual, and in accordance with the needs of the individual and the time schedule of administration of the other factors. KGF may administered
10 at any effective time before entrance of liver cells into S-phase is desired. KGF administration may also continue thereafter. Generally, however, KGF may be administered between about 0 and about 28 days before entrance of liver cells into S-phase is desired. Preferably KGF is administered between about 6 hours and about 14 days before entrance of liver cells into S-phase is desired. Most preferably, KGF is administered as a single bolus between about 24 hours and
15 about 8 days before entrance of liver cells into S-phase is desired. More than one administration of KGF may be desirable in accordance with the needs of the individual as determined by the prescribing physician or veterinarian, and KGF may be administered any effective amount of times. Successive administrations generally can be performed at intervals ranging from about hourly to about weekly, but are preferably done at about daily intervals, or by continuous
20 infusion. KGF administration may continue for any effective time after liver cells have begun to proliferate, but preferably for not longer than about 12 days thereafter. KGF may be administered in any effective daily dosage. In general, however, daily dosages ranging from about 5 µg/kg to about 20 mg/kg. Preferably, KGF is administered in daily dosages ranging

from about 100 µg/kg to about 10 mg/kg. Most preferred is the administration of daily dosages of about 1 mg/kg of KGF. KGF may be administered by any effective route. It may be administered, for example, IV, IM, intraperitoneally, directly into the liver, subcutaneously, orally, by suppository, or by production *in situ* in the liver after liver transduction with an effective vector with a gene construct for KGF expression, or the like, for example an adenovirus vector, or the like. Subcutaneous administration as a bolus is preferred. For subcutaneous administration, KGF may be dissolved in any effective buffer. For example lyophilized KGF may be reconstituted in a 0.01% by weight polyoxyethylenesorbitan monolaurate solution (Sigma) (TWEEN 20), or any other effective buffer. Other suitable buffers are well known in the art.

A triple combination of T3, KGF and hepatocyte growth factor (HGF) was also tried. The cell proliferating characteristics, and the transduction efficiency of the triple combination was not statistically significantly different from the T3/KGF combination, although in some experiments the total number of cells induced to proliferate and liver cells transduced may have been slightly higher.

In accordance with the present invention, the factors may be administered in any effective order or time interval. However, the factors are preferably "concurrently administered," meaning that independent of the order in which the factors are administered, the factors are administered within a time interval such that the effect of the factors on the proliferation of liver cells is at least greater than additive. The factors may also be administered together. Preferably, dividing liver cells in a quantity about 10% or greater than the sum of the quantity of cells that would have resulted had the factors been independently administered will result from concurrent administration. Most preferably, dividing liver cells in a quantity about 30% or greater than the

sum of the quantity of liver cells that would have resulted had the factors been independently administered will result from the concurrent administration.

In accordance with the present invention, each factor may be independently administered any effective number of times, including more than once, as may be indicated by a physician or
5 veterinarian.

In accordance with the present invention, the factors may be administered in any effective amount. However, preferably administration of an "effective amount" will be an amount of each factor such that when administered in combination with the other factor or factors the effect of the factors on the induction of *in vivo* liver cell proliferation is synergistic. In accordance with
10 the present invention, the amount of each factor administered is such that the effect of the factors on the proliferation of liver cells is at least greater than additive. Preferably, proliferating liver cells in a quantity about 10% or greater than the sum of the quantity of liver cells that would have resulted had the factors been independently administered in those amounts will result from the concurrent administration of an effective amount of each factor. Most preferably,
15 proliferating liver cells in a quantity about 30% or greater than the sum of the quantity of liver cells that would have resulted had the factors been independently administered in those amounts will result from the concurrent administration of an effective amount of each factor.

The utility of the administration of any particular amount of each factor and the time and regimen for the administration of the different factors will vary according to several variables,
20 including the species, weight, and other variables, and must be evaluated empirically. It is within the skill of the art to modify the procedure and produce the desired effect in a specific subject.

Several liver-directed gene transfer vectors may be used in accordance with the present invention, including retroviral vectors, adeno-associated virus vectors, lipofectant agents,

receptor based transfer, and combinations thereof. See Ferry & Heard, Human Gene Ther. 9:1975-1981 (1998). Preferred are retroviral vectors. The production and use of retroviral vectors as gene transfer vectors is well documented in the literature, including the articles by Mann, et al., Cell 33:153-159 (1983), Cosset, et al., J. Virol. 69(12):7430-7436 (1995), which are
5 hereby incorporated by reference in their entirety. Production of retroviral vector as described by Cosset, et al., is preferred. Most preferred is the administration of a retrovirus complexed with cationic liposomes, in particular the cationic liposome DiOctadecylamidoGlycylSpermine (DOGS). Infectivity of retroviral vectors has been found to be increased by the addition of DOGS to the retroviral vector preparations.

10 In accordance with the present invention, a liver-directed vector is preferably administered such that it transduces the liver cells within a few hours of maximal liver-cell division. However, the vector may be administered prior to or after that time, as may be more effective, depending on the mode of administration of the vector, the species, age, condition, and in accordance with the needs of the individual as determined by the prescribing physician or
15 veterinarian. Generally, however, the vector may be administered between about 0 and about 28 days after administration of the factors. Preferably the vector is administered between about 6 hours and about 14 days after factor administration. Most preferably, the vector is administered between about 24 hours and about 8 days after factor administration. More than one administration of vector may also be desirable in accordance with the needs of the individual as
20 determined by the prescribing physician or veterinarian. These successive administrations generally can be performed at intervals ranging from hourly to weekly, but are preferably done at between about 6 and about 24 hour intervals.

The method of administering the liver-directed vector to the liver contemplated in accordance with the present invention will vary in accordance with the needs of the individual as determined by the physician or veterinarian. In general, however, *in vivo* liver transduction may be performed according to a modified method described in Rettinger, et al., J. Surg. Res. 54:418-425 (1993), which is hereby incorporated by reference in its entirety. The modified method of Rettinger is preferred. Subjects are anesthetized, and a laparotomy is performed. The portal vein and hepatic artery are clamped using micro-aneurysm clips, or the like, and the portal vein is cannulated with a needle having an appropriate gauge (according to the size of the subject) proximal to the clip and retroviral supernatant is injected. Hemostasis may be achieved by direct pressure to the portal vein and topical thrombin application, or the like. The clips are removed and the abdomen sutured, and the subjects are allowed to convalesce. See Forbes, et al., Gene Therapy 5:552-555 at 554 (1998), which is hereby incorporated by reference in its entirety.

Alternatively, the procedure may be accomplished with portal injection of the liver-directed vector, in which case the portal vein is cannulated and the vector solution pumped into the portal vein over a period of time. See, e.g., Kay, et al., Human Gene Therapy 3:641-647 at 642 (1992) (using 1 ml of viral supernatant in 8 µg/ml Polybrene infused over 50 minutes in mice), which is hereby incorporated by reference in its entirety.

Alternatively, the vector is delivered by asanguineous perfusion of the liver, whereby the liver is selectively perfused after excluding normal blood flow from the liver. This may be accomplished by clamping the hepatic artery, portal vein, suprahepatic vena cava, right suprarenal vein and infrahepatic vena cava to achieve the total vascular exclusion of the liver. The portal vein is then cannulated with a catheter of appropriate gauge, while an incision is made in the anterior wall of the infrahepatic vena cava, with a suction cannula placed into the vena

cava to collect outflow. Liver perfusion, preferably single pass perfusion, is then performed using a pump. Flow rate may range from 0.1 ml/min per gram of liver to 10 ml/min per gram of liver. A rate of 1 ml/min per gram of liver is preferred, and may be increased to ensure satisfactory perfusion of the organ. After perfusion, the portal and caval vein incisions are
5 sutured and the liver is revascularized. Cardoso, et al., Human Gene Therapy 4:411-418 at 412 (1993), which is hereby incorporated by reference in its entirety.

Alternatively, another preferred method of vector administration is by peripheral vein intravenous injection. The vector may be administered by intravenous injection any number of effective times, including more than once, as may be indicated by a physician or veterinarian.
10 The intravenous injection may be administered at any effective time, as described *supra*, but is most preferably administered 18 - 36 hours after the administration of the growth factors.

Infectivity of liver-directed vectors, in particular retroviral vectors, and hepatic transfection efficiency can be increased by the addition of liposomes, preferably cationic liposomes, to the liver-directed vector preparations. The cationic liposome
15 DiOctadecylamidoGlycylSpermine (DOGS) is preferred. DOGS is effective in concentrations of about 1 to about 30 μ g per ml. Preferred is a final DOGS concentration of about 5 μ g per ml. The titer of vector/DOGS solution administered has preferably about 1×10^9 i.u./ml after ultrafiltration.

Any effective volume of vector may be administered. The volume to be administered
20 will depend on the i.u./ml of vector preparation, and the total number of i.u. it is desired to administer. In general, however, given a vector preparation having 1×10^9 i.u./ml, about 0.1 ml of vector preparation per kg of subject weight to about 80 ml of vector preparation per kg of subject weight may be administered. Most preferred is the administration of about 8 ml of a solution of

1x10⁹ i.u./ml per kg of subject weight. Although any effective amount of total i.u. may be administered, preferably a total of about 8x10⁹ i.u. per kg of subject weight are administered.

Examples of conditions which are effectively treated with the combinations and methods of the present invention include treatment and prevention of any conditions that may be prevented or ameliorated by the proliferation of liver cells, including liver cell deficiencies, cirrhosis of the liver, and the like. Other conditions which are effectively treated with the combinations and methods of the present invention include treatment and prevention of any conditions that may be prevented or ameliorated by the expression in the liver of RNAs, polypeptides or proteins, or combinations thereof, for action in the liver or secretion of polypeptides or proteins into the bloodstream or the gastrointestinal tract.

The liver directed vector includes a nucleic acid engineered such that it encodes the RNA, protein or polypeptide to be expressed, operably linked to a sequence, such as a promoter or the like, and alternatively also an enhancer or the like, such that the encoded RNA, or mRNA for the polypeptide or protein, will be transcribed in the liver cells. If a protein or polypeptide is to be expressed, the nucleic acid is preferably engineered such that when the mRNA is transcribed it has a sequence such that ~~the~~ it may be translated. See, e.g., Ill, et al., Blood Coagulation and Fibrinolysis 8(Suppl 2):S23-S30 (1997)(incorporated herein by reference in its entirety).

The methods and compositions described herein find particular utility in the expression in the liver of RNAs, proteins and polypeptides known to have a therapeutic effect, including, but not limited to, for example, afamin (see, e.g., United States Patent Nos. 5,652,352 and 5,767,243, hereby incorporated by reference including drawings); anti-inflammatory CD14 peptides (see, e.g., United States Patent No. 5,766,593, hereby incorporated by reference including drawings); art (see, e.g., United States Patent No. 5,766,877, hereby incorporated by reference including

drawings); erythropoietins (see, e.g., United States Patent Nos. 4,703,008, 5,441,868, 5,618,698, 5,547,933, 5,621,080 and 5,756,349, hereby incorporated by reference including drawings); granulocyte colony-stimulating factor (see, e.g., United States Patent Nos. hereby incorporated by reference including drawings); granulocyte-colony stimulating factors (see, e.g., United States Patent Nos. 4,810,643, 4,999,291, 5,581,476, 5,582,823 (human) and 5,606,024 (canine) and PCT Publication Nos. 91/05795, 92/17505 and 95/17206, hereby incorporated by reference including drawings); interferon consensus (see, e.g., United States Patent Nos. 5,661,009, 5,372,808, 5,541,293, 4,897,471, and 4,695,623, hereby incorporated by reference including drawings); interleukins (see, e.g., United States Patent Nos. 5,075,222, hereby incorporated by reference including drawings); leptin (OB protein) (see, e.g., PCT Publication Nos. 96/40912, 96/05309, 97/00128, 97/01010 and 97/06816, hereby incorporated by reference including drawings); NDF peptides (see, e.g., United States Patent No. 5,670,342, hereby incorporated by reference including drawings); platelet-derived growth factor B (see, e.g., United States Patent No. 5,428,135, 5,272,064 and 5,149,792 hereby incorporated by reference including drawings); progenitor B cell stimulating factor (see, e.g., United States Patent No. 5,580,754, incorporated herein by reference in its entirety); stem cell factor interleukin-6 (see, e.g., United States Patent No. 5,610,056, hereby incorporated by reference including drawings).

The construct may include sequences encoding proteins engineered such that they will be secreted (see, e.g., United States Patent No. 5,541,083, hereby incorporated by reference including drawings; von Heijne, J. Mol. Biol. 184:99-105 (1985) and references therein, incorporated herein by reference; Rusch & Kendall, Mol. Memb. Biol. 12:295-307 (1995) and references therein, incorporated herein by reference).

In addition, other RNAs, proteins and polypeptides that may be expressed include, but are

not limited to insulin; gastrin; prolactin; adrenocorticotrophic hormone (ACTH); luteinizing hormone (LH); follicle stimulating hormone (FSH); human chorionic gonadotropin (HCG); motilin; interferons (alpha, beta, gamma); tumor necrosis factor-binding protein (TNF-bp); interleukin-1 receptor antagonist (IL-1ra); brain derived neurotrophic factor (BDNF); glial
5 derived neurotrophic factor (GDNF); neurotrophic factor 3 (NT3); fibroblast growth factors (FGF); neurotrophic growth factor (NGF); bone growth factors such as osteoprotegerin (OPG); insulin-like growth factors (IGFs); macrophage colony stimulating factor (M-CSF); granulocyte macrophage colony stimulating factor (GM-CSF); megakaryocyte derived growth factor (MGDF); keratinocyte growth factor (KGF); thrombopoietin; platelet-derived growth factor
10 (PGDF); colony stimulating growth factors (CSFs); bone morphogenic protein (BMP); superoxide dismutase (SOD); tissue plasminogen activator (TPA); urokinase; streptokinase; kallikrein; interleukin genes; cystic fibrosis transmembrane conductance regulator; human factor VII; interleukin receptor antagonist proteins; cytokines; 4-hydroxyphenylpyruvic acid dioxygenase; tissue-type plasminogen activator or plasminogen; transferlpha-fetoprotein and
15 albumin genes; glucuronosyltransferase; thrombopoietin; immunoregulatory cytokines; phenylalanine hydroxylase; ribozymes; dominant negative genes, and the like; cytochrome genes; immunoglobulin A (Busch, et al., Gastroenterology 115(1):129-38 (1998) incorporated herein by reference); canalicular bile salt transporters; bilirubin uridine diphosphate glucuronosyltransferase; multidrug resistance genes; organic anion transporters; bile salt
20 transporters; ABC transporters, and the like, and other membrane proteins including transporters, receptors, cell adhesion molecules, and the like.

The term protein, as used herein, includes peptides, polypeptides, consensus molecules, analogs, derivatives or combinations thereof. Also included are those proteins with amino acid

substitutions which are "conservative" in that they do not affect the intended function of the protein. See generally, Creighton, Proteins, W.H. Freeman and Company, N.Y., (1984) 498 pp. plus index, *passim*.

Other conditions that may be treated by the compositions and methods of the present invention include inborn errors of metabolism; lymph and blood protein and polypeptide deficiencies or overexpression; cancer (see, e.g., Zhang, W.-W., J. Mol. Med. 74:191-204 (1996)); the several forms of hepatitis and other infectious diseases affecting the liver; hypertension; clotting abnormalities including hemophilia A (Factor XIII deficiency), hemophilia B (Factor IX deficiency) and proteins C and S; obesity; hypercholesterolemia; cystic fibrosis; alpha 1-antitrypsin deficiency; cirrhosis of the liver; diabetes; ornithine transcarbamylase deficiency and other urea cycle related conditions (see Batshaw, Ann. Neurol. 35(2):133-141 (1994)); tryosinemia; phenylketonuria (see Eisensmith & Woo, J. Inher. Metab. Dis. 19:412-423 (1996)); haemochromatosis; carbohydrate disorders, including for example, the several glycogen storage diseases (including glucose-6-phosphatase deficiency); galactosemia and fructose intolerance; lysosomal storage deficiencies; CPS deficiency; FAH deficiency; propionic acidemia; methylmalonic acidemia; defects of branch amino acid degradation (maple syrupe urine disease); lipid metabolism, including LDL receptor deficiency (see Li, et al., J. Clin. Invest. 95:768-773 (1995)), and expression of apoA1 gene for increases in serum HDL levels; porphyrin metabolism by adding porphobilinogen deaminase activity; Wilson disease (copper-transporting ATPase); hyperbilirubemias, including Crigler-Najjar syndrome and Gilbert Syndrome (UDP-glucuronosyltransferase); Dubin-Johnson syndrome (cMOAT); expression of hammerhead ribozymes to cleave hepatitis C virus RNA and inhibit viral protein translation; expressing dominant negative genes, including for example mutants of the hepadnaviral core

protein as antiviral agents; correction of Protein C deficiency; correction of Factor X deficiency;
expression of antisense RNA, for example anti-sense RNA complementary to hepatitis B virus to
inhibit viral proliferation, or to other protein to inhibit its expression; correction of
citrullinaemia; expression of p53 to inhibit tumor progression; expression of leptin for obesity;
5 expression of Alpha 1-antitrypsin to treat emphysema; expression of apolipoprotein B mRNA-
editing complex to reduce LDL levels; inflammatory bowel disease by expressing
immunoregulatory cytokines; attenuation of hypertension and cardiac hypertrophy and renal
injury by expression of Kallikrein gene; treatment of tyrosinemia type I; treatment of diabetes by
expression of insulin gene, in particular under a glucose responsive promoter/enhancer; treatment
10 of hyperlipidemias associated with apolipoprotein E and LDL receptor deficiencies by
expression of human lipoprotein lipase; expression of phenylalanine hydroxylase for
phenylketonuria; treatment of hypertriglyceridemia and impaired fat tolerance by expression of
lipoprotein lipase; cancer gene therapy; correction of mitochondrial enzyme deficiencies;
treatment of mucopolysaccharidosis type VII; secretion of cloned immunoglobulins, including
15 antibodies; treatment of alpha-galactosidase A deficiency and Fabry disease; treatment of
dwarfism by expression of growth hormone; treatment of tetrahydrobiopterin deficiency in
hyperphenylalaninemic patients by gene transfer of 6-pyruvoyl-tetrahydropterin synthase;
correction of diabetic alterations by glucokinase; treatment of type I hyperoxaluria; expression of
the enzyme apobec-1 for hypercholesterolemia in LDL receptor-deficiency; treatment of familial
20 hypercholesterolaemia by expression of the VLDL receptor gene; expression of human pyruvate
kinase (PK) for gene therapy of human PK deficiency; correction of methylmalonyl-CoA mutase
deficiency; treatment of phenylalanine hydroxylase deficiencies; adenosine deaminase and purine
nucleoside phosphorylase deficiencies, and the like.

The methods and compositions of the present invention are also applicable for *in vivo* gene immunization, by direct expression of antigens in the liver, for example.

The present invention also finds applications in animal sciences and for veterinary uses. For example, the present invention is applicable in expressing RNA, proteins and polypeptides in the liver of animals - for action within the liver, or for secretion into the circulation or gastrointestinal tract. The long-term expression, inducible expression, or responsive expression of growth hormone, or growth hormone releasing substances like growth hormone releasing hormone, for example, in adult lactating cows may be achieved without causing the adverse consequences that the constitutive expression of the gene products may have in calves. Similarly, for example, the expression of leptins in farm animals an effective amount of time prior to slaughter for the production of leaner meat, without the adverse consequences such expression would have had had the leptins been expressed in growing and developing animals. The expression of major histocompatibility complex (MHC) molecules, and the like, in cells of the liver of an animal may be altered, for example, by expression of specific MHC molecules, or by inhibition of expression of MHC molecules by anti-sense RNA expression prior to organ transplantation. The methods and compositions may also be used in avian species, for example to produce avian growth hormones, or the like (see, e.g., United States Patent Nos. 5,151,551 and 5,162,215, incorporated herein by reference in their entirety, including figures). The sequence of the RNAs, polypeptides or proteins encoded by the liver directed gene transfer vector is preferably conspecific.

The combination of factors of the present invention may also be used to enhance the proliferation and transduction of liver cells *in vitro*.

EXAMPLES

Unless otherwise specified, the following procedures were used throughout the following examples.

Male Wistar rats weighing 200-250 grams were used. They were housed in a temperature and light controlled room (12 hour light/dark cycle) and were allowed water and food ad libitum.

T3 (Sigma, St. Louis, MO USA), 4 mg per kilogram of body weight was dissolved in 100 μ l of 0.01 M NaOH in 0.9 M NaCl and administered by subcutaneous injection in the flank region of the animal.

Lyophilized KGF (full lenght recombinant KGF, AMGEN, Thousand Oaks, CA) 1 mg per kilogram of body weight was dissolved in 100 μ l of a suitable buffer (0.01% by weight polyoxyethylenesorbitan monolaurate (TWEEN 20, Sigma) solution) and administered by subcutaneous injection in the flank region of the animal.

Measurement of liver cell proliferation was performed by BrdU labeling. BrdU (Sigma) (50 mg/kg dissolved in 800 μ l of 0.9% NaCl) was injected intraperitoneally 1 hour prior to sacrifice. Livers of the animals were removed and fixed in 60% methanol, 30% chloroform and 10% glacial acetic acid before routine histological processing and embedding in wax blocks. The incorporation of BrdU into hepatic nuclei was detected in 4 μ m thick sections using a mouse monoclonal antibody (Dako, Glostrup, Denmark), followed by a biotinilated rabbit anti-mouse secondary antibody (Dako). Visualization was achieved by the indirect peroxidase technique using diaminobenzidine tetrahydrochloride dihydrate (Aldrich Chemical, Milwaukee, WI USA) as a substrate. The labeling index and percentage of hepatic mitoses was determined by counting 2000 hepatocytes per section from consecutive light microscopy fields. See Forbes, et al., Gene Therapy 5:552-555 at 553 (1998)(incorporated herein by reference in its entirety).

In vivo liver transduction was according to a modified method described in Rettinger, et al., J. Surg. Res. 54:418-425 (1993). Animals were anesthetized with isoflurane. A midline laparotomy was performed, the bowel was deflected to the left and wrapped in normal saline-soaked gauze. The portal vein and hepatic artery were clamped using micro-aneurysm clips, the portal vein was cannulated with a 26-gauge needle proximal to the clip and 1 ml of retroviral supernatant was injected over 5 minutes. Hemostasis was achieved by direct pressure to the portal vein and topical thrombin (Sigma) application. The clips were removed and the abdomen sutured, and the animals were allowed to recover in a warm, ^{quite} ~~quite~~ environment. See Forbes, et al., Gene Therapy 5:552-555 at 554 (1998). For peripheral vein injection, 1 ml of the retrovirus/DOGS complex was injected via tail vein as a bolus.

Transduction of liver cells was measured by β -galactosidase activity. Animals were anesthetized and sacrificed by exsanguination. The liver was removed and frozen in liquid nitrogen. Ten-micron frozen sections were cut on to glass slides, fixed in a solution of 1.25% glutaraldehyde at 4°C, washed with phosphate buffered saline (PBS) then covered in X-gal (Gibco BRL, Grand Island, NY) solution (1 mg/ml, pH 7.4) and incubated overnight at 37°C. Sections were then washed in PBS, counterstained lightly with eosin or carmalum stain, dehydrated and mounted in pertex (Cellpath, Hemel Hempstead, UK). The proportion of hepatocytes expressing nuclear β -galactosidase was calculated by counting 2000 hepatocytes per section from three separate areas.

High titer amphotropic retroviral vector stocks were generated by the method described in Cosset, et al., J. Virol. 69:7430-7436 (1995). TELCeb/AF-7 cells were used to generate high titers of amphotropic retrovirus vector stock. This is a high titer replication defective vector encoding the gene for β -galactosidase together with a nuclear localization signal. Cells were

grown in DMEM supplemented with 10% fetal calf serum at 37°C. To prepare viruses, cells were grown to confluency and refed with fresh medium overnight. The following day, virus containing supernatants were harvested and filtered through 0.4 µm filters for determination of retrovirus titer and helper virus assay. Retrovirus titer and helper virus assay were determined on NIH3T3 cells as previously described using dilutions of virus. In the presence of polybrene a titer of 1×10^7 I.U./ml was established. To generate virosome complexes a 20 µl aliquot of virus stock was added to 0.5 ml of serum free DMEM plus the appropriate amount of amphiphile and incubated for 30 minutes at room temperature to allow complex formation to occur. For titer estimation each respective complex was prepared in 3 ml with 10% serum containing medium then added to NIH3T3 cells and left for 24 hours for infection to occur.

Example 1

Induction of liver cell proliferation - dose ^{response} ~~response~~ curves for individual factors.

Dose response curves were initially performed to identify the dose at which KGF and T3 independently cause maximum hepatocyte proliferation at 24 hours. KGF at 1 mg/kg injected subcutaneously was found to produce maximal proliferation. T3 at 4 mg/kg injected subcutaneously was found to produce maximal proliferation.

Example 2

Induction of liver cell proliferation - time course for individual factors.

The times after injection having maximum liver cell proliferation (as measured by DNA synthesis), were also independently established for each of the individual factors. An injection corresponding to the dose producing maximal cell proliferation, as established in Example 1, was independently administered to 2 groups of subjects, one group for each factor. Dividing cells were labelled by administering BrdU 1 hour prior to sacrifice. At 6, 9, 12, 15, 18, 24, and 30

hours after injection three animals of each group were sacrificed and labelled liver cells counted. As shown in figure 1, 1 mg/kg KGF injected subcutaneously was found to produce maximal cell proliferation, corresponding to approximately 9%, 24 hours after injection. As shown in figure 2, 4 mg/kg T3 injected subcutaneously was found to produce maximal cell proliferation, corresponding to approximately 10%, 24 hours after injection. KGF was found to produce cell proliferation in all zones of the liver. T3 was found to produce cell proliferation predominantly in the mid-zonal region.

Example 3

Unexpected liver cell proliferation effects found to result from administration of combination.

While searching for a manner of producing a clinically acceptable level of liver cell proliferation without a need for prior injury to the liver, a series of experiments were performed whereby a combination of factors was administered to subjects and liver cell proliferation measured 24 hours after administration. Three factor combinations were independently tested, each combination in a different group of animals. A first group received 4 mg/kg T3 at 0 hours. A second group received 1 mg/kg KGF at 0 hours. A third group received 1 mg/kg KGF and 4 mg/kg T3 at 0 hours. A fourth group served as control and received the vehicle or diluent only.

The results of the experiment are shown in figure 2. Following a single injection of T3, approximately 10% of hepatocytes are induced into S-phase at 24 hours. Similarly, following KGF injection, approximately 9% of hepatocytes are proliferating at 24 hours. The combination of T3 and KGF results in approximately 35% proliferating hepatocytes at 24 hours.

These *in vivo* tests showed significantly improved liver cell proliferation when a combination of T3 and KGF are administered, when compared to liver cell proliferation levels resulting from the administration of individual factors. In particular, the increased proportion of

liver cells proliferating when a combination of T3 and KGF is administered has been found to be much greater than would have been predicted given the known *in vivo* proliferative response of liver cells to the individual growth factors. Administration of a combination of factors yielded a semi-synchronous wave of liver cell proliferation involving a significantly larger extent of liver cells compared to the administration of a similar amount of individual factors when administered independently. These improvements are surprising and valuable, for they provide *in vivo* liver cell proliferation in a range that is clinically useful and substantially equivalent to that obtained by partial hepatectomy, but without a need to cause prior injury to the liver.

Example 4

Cumulative hepatic proliferation.

Cumulative hepatic proliferation resulting from co-administration of T3 and KGF was determined as follows. At 0 hours, 4 mg/kg T3 and 1 mg/kg KGF were administered by subcutaneous injection into a group of 3 rats. A control group of 2 animals received injections of the diluents only. Animals were labelled every 5 hours with BrdU between hours 12 and 32. The animals were sacrificed at 33 hours and incorporation of BrdU in liver cells was measured. The results of the experiment are shown in figure 3. Animals administered T3 and KGF had approximately 60% of their liver cells labelled with BrdU. Notably, significant biliary cell proliferation was also observed.

Example 5

Induction of liver cell proliferation *in vivo* by administration of combination of factors results in enhanced transduction of liver cells by retroviral vectors.

Having found an effective method of inducing liver cell proliferation within the intact liver, it was next investigated whether a rise in *in vivo* retroviral transduction corresponding to the increased proliferation of liver cells would result. At hour 0, several groups of four rats

received an administration of either a single growth factor (either 4 mg/kg T3 or 1 mg/kg KGF) or the combination of 4 mg/kg T3/ 1 mg/kg KGF. A control group of three rats received injections of the growth factor diluents only. At 24 hours all rats were injected intraportally with 1 ml of retrovirus during temporary hepatic artery and portal vein occlusion. All rats were killed 9 days following vector administration. As seen in figure 4, increased cellular proliferation when a combination of factors is administered gives rise to an unexpectedly greater than corresponding rise in the *in vivo* retroviral transfection efficiency. It was found that if the liver was primed by a single growth factor prior to intraportal vector administration then between $1.2 \pm 0.4\%$ and $2.3 \pm 0.5\%$ of hepatocytes were transfected. Following T3 administration the transfected hepatocytes were mostly found in the mid-zonal region. Following KGF administration the transfected hepatocytes were found in all zones of the liver. Following the combined administration of T3 and KGF, the proportion of hepatocytes transfected rose to approximately 7% and these were found throughout all zones of the liver.

The proportion of liver cells transduced following administration of a combination of T3 and KGF was significantly higher than expected, even after taking into account the greater proportion of proliferating cells. While a single injection of T3 induces approximately 10% of liver cells into S-phase at 24 hours, with approximately 1.2% of liver cells being transduced (a ratio of 8.3:1 infectivity), and while after a KGF injection, approximately 9% of hepatocytes are proliferating at 24 hours, with approximately 1% of liver cells being transduced (a ratio of 9:1 infectivity), the combination of T3 and KGF results in approximately 35% proliferating liver cells at 24 hours, with approximately 7% of liver cells being transduced, a superior and unexpected infectivity ratio of 5:1.

Example 6

We have also found that the retroviral construct can be administered by the more clinically acceptable peripheral vein intravenous route during liver hyperplasia and still effectively transfect the liver, and that the efficiency of transfection is increased with multiple
5 administrations of the vector.

The effects of multiple administrations of retroviral vector and the efficacy of using the more clinically acceptable peripheral vein intravenous administration for the retroviral vector were tested. Several groups of 4 animals received either T3 (4 mg/kg), KGF (1 mg/kg), or T3/KGF at 0 hours. Two control animals were injected with growth factor diluent only. All
10 animals received 3 tail vein injections of 1 ml of the TELCeB/AF-7 vector at 18, 24 and 30 hours and were killed on day 10. Hepatic expression of nuclear localized β -galactosidase was quantified. The animals that had received the combination growth factor prior to the vector administration had the most efficient transfection with 6+% of their hepatocytes expressing the transgene throughout all zones of the liver, except the peri-venous area. (Figure 5).

Thus, combinations and methods for inducing a semi-synchronous wave of liver cell
15 proliferation *in vivo* are disclosed. Also disclosed are compositions and methods for enhancing *in vivo* liver cell transduction by liver directed gene transfer vectors. While embodiments and applications of this invention have been shown and described, it will be apparent to those skilled in the art that many modifications are possible without departing from the inventive concepts
20 herein. For example, while the example describes the expression of a marker gene, β -galactosidase, *in vivo* in the liver of experimental animals, those of skill in the art will recognize that different RNAs, including ribozymes and anti-sense RNAs, polypeptides and proteins may be expressed in the liver of a variety animals. Proteins and polypeptides may be secreted into the

gastrointestinal tract by expression in the biliary tract, or into the blood stream by expression in the liver, if the protein or polypeptide have appropriate, well known in the art, leader sequences. As will be recognized by those of skill in the art, different vectors, and in particular different retroviral vectors may be used with the present invention. Those of skill in the art will also recognize that other promoters and enhancers, including constitutive, liver specific, biliary cell specific or hepatocyte specific promoters, enhancers or combinations thereof may be used, including viral promoters and enhancers like those from the several forms of hepatitis virus, and the like, liver specific promoter/enhancers, or promoters/enhancers from liver specific genes. Inducible promoters/enhancers are also well known in the art and may be used. Alternatively promoters/enhancers that are responsive to internal signals, like sex hormones, sugar levels, circadian rhythm signals, and the like may be used. Moreover, expression may be restricted to biliary tract cells, hepatocytes, or both, in particular by use of appropriate promoter/enhancer combinations. Expression may be of any RNAs, proteins or polypeptides, naturally occurring or engineered, including dominant negative effect proteins, which may be accommodated by the liver-directed gene transfer vector and that is capable of being transcribed, and if necessary translated by liver cells. The gene construct may also include introns, poly-adenylation signals, and the like, known to affect the half-life of RNA, and/or its level of expression, or to permit another level of regulation. The invention, therefore is not to be restricted except in the spirit of the appended claims.